Plant Physiology and Biochemistry 140 (2019) 151–157

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Na^+ compartmentation strategy of Chinese cabbage in response to salt stress

Juan Wang[a], Nianwei Qiu[a,*], Ping Wang[a], Weirong Zhang[a], Xiaoying Yang[a], Min Chen[b], Baoshan Wang[b], Jingkuan Sun[c,**]

[a] College of Life Sciences, Qufu Normal University, Qufu, Shandong, 273165, China
[b] Shandong Provincial Key Laboratory of Plant Stress, Shandong Normal University, Jinan, 250014, China
[c] Shandong Provincial Key Laboratory of Eco-Environmental Science for Yellow River Delta, Binzhou University, Binzhou, Shandong, 256600, China

ARTICLE INFO

Keywords:
Chinese cabbage
Na^+ compartmentation
Na^+/H^+ antiporter (NHX)
Gene expression
Salt-tolerance

ABSTRACT

Na^+/H^+ antiporter (NHX), responsible for counter-transport of Na^+ and H^+ across membranes (Na^+ compartmentalization), plays a central role in plant salt-tolerance. In order to explore the Na^+ compartmentalization modes and salt tolerance strategy in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*), the seedlings of a salt-susceptible cabbage cultivar (Kuaicai 38) and a salt-tolerant cabbage cultivar (Qingmaye) were exposed to 100–400 mM NaCl for 30 days. Both of these cultivars showed a gradual decrease in fresh weight and water content and an increase in root-shoot ratio with the increasing NaCl-treatment concentration. The distribution of Na^+ in these two cultivars was similar, with the green leaves showing the highest Na^+ content, followed by inflated midribs, stems, and roots. The Na^+ concentration in the apoplast was higher than that in the protoplast of the leaves. The expression levels of *BrNHX1-1* and *BrNHX1-2* in the leaves of Qingmaye were the highest among all *BrNHX* members, and increased after salt treatment. However, only *BrNHX1-1* was expressed in Kuaicai 38. These results indicate that Na^+ compartmentation into vacuoles is the major salt-adaptation strategy in Chinese cabbage. Coordinated overexpression of *BrNHX1-1* and *BrNHX1-2* may confer greater salt-tolerance for Chinese cabbage.

1. Introduction

Saline soil is widely distributed all over the world, and more than 800 million ha soil are negatively impacted by salinity (FAO, 2009). The cultivation of salt-tolerant vegetable crops is an important and a feasible way to develop and utilize saline soil, because vegetable crops have a higher economic value than that of grain crops (Zhang et al., 2014). Chinese cabbage (*Brassica rapa* L. ssp. *Pekinensis*) is one of the most important vegetables in East Asia. It has been documented that although salt treatments with 100–400 mM NaCl negatively influence seed germination, seedling growth, leafy head yield and fruit formation, Chinese cabbage can long live under 200 mM NaCl (Qiu et al., 2015). Chinese cabbage has no salt glands and cannot secrete salt under salt stress (Yuan et al., 2016). In addition, it cannot effectively prevent salt from entering the plant body or dilute the salt by absorbing a large amount of water (Zhang et al., 2014; Yuan et al., 2019). At present, there are a few reports on salt tolerance studies of *Brassica* crops using molecular markers and transcriptomics (Kumar et al., 2015; Saha et al., 2015; Qiu et al., 2017; Zhang et al., 2018). However, the salt tolerance mechanism of Chinese cabbage is still poorly understood.

In order to survive salt stress, plants have evolved a variety of stress-resistance mechanisms, including the increase of antioxidant capacity and compatible osmolytes, the decrease of sodium absorption, and the compartmentalization of Na^+ away from the cytoplasm (Deinlein et al., 2014; Tang et al., 2015; Shah et al., 2018). Na^+/H^+ antiporter (NHX), responsible for counter-transport of Na^+ and H^+ across membranes (Na^+ compartmentalization), plays a central role in plant salt-tolerance (Munns and Tester, 2008; Hasegawa, 2013). To prevent excessive Na^+ accumulation in plant cytoplasm, two types of *NHX* genes could be used for Na^+ compartmentalization: plasma membrane Na^+/H^+ antiporter genes and vacuolar Na^+/H^+ antiporter genes (Dong et al., 2018). Up-regulation of Na^+/H^+ antiporters is a common feature of all halophytes and salt-tolerant crops under salt stress (Chen et al., 2017; Dong et al., 2018). Overexpression of both types of the *NHX* genes can enhance salt tolerance of transgenic *Brassica* species (Saha et al., 2015). The *NHX* clade is represented by eight genes in *Arabidopsis thaliana* (*AtNHX1 – 8*). AtNHX1 and AtNHX2 have been well established to be the tonoplast Na^+/H^+ antiporters, and AtNHX7 is the plasma membrane Na^+/H^+ antiporters. AtNHX2 – 6 are phylogenetically linked to AtNHX1, while AtNHX8 is related to AtNHX7 (Yokoi et al., 2002; An et al., 2007). Up to

---

[*] Corresponding author.
[**] Corresponding author.
E-mail addresses: nianweiqiu@163.com (N. Qiu), sunjingkuan@126.com (J. Sun).

https://doi.org/10.1016/j.plaphy.2019.05.001
Received 2 April 2019; Received in revised form 30 April 2019; Accepted 2 May 2019

2013, the DNA sequences of NHX proteins had been identified in more than 60 plant species (Xu et al., 2013). However, the *NHX* genes in Chinese cabbage and their functions are yet to be understood.

In this study, we identified eight *NHX* gene members in Chinese cabbage through genome-wide bioinformatics analysis. The expression pattern of these *NHX* genes in the leaves of two Chinese cabbage varieties with different salt tolerance capacities was characterized in response to salt stress. At the same time, combined with the distribution of Na^+ in different organs, the strategy of Na^+ compartmentation of Chinese cabbage in adaptation to the saline environment was analyzed.

2. Materials and methods

2.1. Plant culture and treatments

A salt-susceptible cabbage cultivar (Kuaicai 38, Tianjin Shennong Seed Co., LTD; KC-38) and a salt-tolerant cabbage cultivar (Qingmaye, Shandong Weifang Seed Co., LTD; QMY) were selected from eighteen varieties of Chinese cabbage widely cultivated in China (Yang et al., 2017). Seedlings of Chinese cabbage were grown in plastic pots (25 cm in diameter and 22 cm in height) filled with quartz sand and moistened with half-strength Hoagland nutrient solution. The seedlings were incubated in a greenhouse with a day/night temperature at 25/15 °C and a maximum photon flux density of approximately 1000 $\mu mol/m^2/s$. Five uniform seedlings were reserved per pot. When the cabbage seedlings had 4-5 leaves, the uniformly sized seedlings were selected and exposed to salt treatment. The salt concentration was gradually increased in an increment of 100 mM NaCl every day until the final concentrations (100, 200, 300, or 400 mM NaCl) were achieved. NaCl was dissolved with Hoagland solution. For the control group (0 mM NaCl treatment), the actual Na^+ concentration was 2 mM in Hoagland solution. All seedlings reached their final salinity levels on the same day. The seedlings were watered daily to drip with approximate 0.5 L of NaCl solution to keep the NaCl concentration steady. The seedlings were harvested 30 days after reaching the final NaCl concentration for analysis of Na^+ compartmentation in green leaves (GL), inflated midribs (IMR), stems (S), and roots (R).

For expression analysis of *BrNHXs* in different tissues, uniformly sized seedlings with three fully opened leaves were directly exposed to 200 mM NaCl. The whole leaves (including green leaves and inflated midribs) were harvested after 0, 6, 12, 24, 48 and 96 h of salt treatment, immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation.

2.2. Fresh weight, root-shoot ratio and water content

The green leaves (GL), inflated midribs (IMR), stems (S) and roots (R) were separated, and the fresh weight (FW) was recorded after a 30-day exposure to 100–400 mM NaCl. The root-shoot ratio was calculated by the formula: FW(GL + IMR + S)/FW(R). A certain amount of fresh plant tissue samples were dried in an oven at 70 °C for 72 h, and the dry weights (DW) were measured. The water content (WC) was calculated by the formula: FW/DW.

2.3. Na^+ content in Chinese cabbage organs

The dry samples (50 mg) of Chinese cabbage organs treated with 100–400 mM NaCl for 30-day were ashed at 500 °C in a muffle furnace. The ash was dissolved in concentrated nitric acid and diluted with distilled water. The Na^+ concentration was measured by a M410 Flame Photometer (Sherwood; the United Kingdom), using NaCl for calibration (0–50 mg/L Na^+). The 410 flame photometer uses a low temperature flame of natural gas to directly measure Na^+ concentration.

2.4. Na^+ concentrations in apoplast and protoplast

After a 30-day exposure to 100 and 200 mM NaCl, the leaves were frozen in advance and then placed into a syringe. The cell sap was squeezed from the leaf tissues with the syringe. Apoplastic sap was collected from the leaves by the method of Tetlow and Farrar (1993). Chinese cabbage leaves were harvested and placed into a plastic syringe, with leaf peri-axle towards the bottom. The syringe was centrifuged at $200 \times g$ (10 min at 4 °C) with its cusp embedded in an Eppendorf tube (1.0 mL) so that the apoplastic sap would flow into the Eppendorf tube under centrifugal force. Apoplastic sap and the liquid squeezed from the leaf tissues (100 μL) was blanched with 200 μL concentrated nitric acid and then diluted with distilled water. The Na^+ concentration was measured using a M410 Flame Photometer.

The Na^+ concentration in protoplasts was calculated as described by Flowers and Yeo (1986), assuming that the volume ratio of apoplast to protoplast liquid was 3:97. The calculation was performed according to the equation: $C_p = (T - C_a \times V_a)/V_p$, where T is the Na^+ content in 1 mL cell sap (equal to the sum of the Na^+ content in apoplast and protoplasts), C_a is the Na^+ concentration in apoplast, V_a is the volume of apoplastic sap, and V_p is the volume of protoplast sap. For use in the above equation, $V_a = V_t \times 3\%$, $V_p = V_t \times 97\%$, $V_t = 1$ mL (cell sap).

2.5. Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from the N_2-frozen leaves with Trizol reagent from Invitrogen (Carlsbad, CA, USA) and then treated with RNase-free DNase I (Promega, WI, USA) for 2 min at 42 °C according to the manufacturer's instructions. First-strand cDNA was synthesized with a PrimeScriptTM RT reagent Kit (Takara, Dalian, China) from 1 μg of total RNA. RT-qPCR was carried out by a SYBR Green Master Mix (Qiagen, Hilden, Germany) with an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The gene-specific primers designed for the *BrNHX* genes are listed in Table 1. The *BrACTIN* gene was used as a constitutive expression control in the RT-qPCR experiments (*BrACTIN-F*: 5′-GCTCAGTCCAAGAGAGGTATTC-3′, *BrACTIN-R*: 5′-GCTCGTTGTAGAAAGTGGATC-3′). The PCR program used was as follows: an initial polymerase activation step of 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 40 s. After each RT-PCR run, a dissociation curve was designed (55°C-95 °C, 0.5 °C/10 s) to confirm the specificity of the product and to avoid the production of primer dimers. The relative amounts of the amplification products were calculated by the comparative $2^{-\Delta\Delta C_T}$ method.

Table 1
Specific primers of *BrNHX* genes for real-time quantitative reverse transcriptase polymerase chain reaction.

BrNHX name	Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)
BrNHX1.1	*Bra036110*	GTCTTTCCCTTGTCGTTTCTCTCA	CTGGTGCGGCAATAGGAATCT
BrNHX1.2	*Bra020599*	CGTCTTCCCATTATCTTTCCTGTC	GTGGTGGCTTTCACAGTGCTG
BrNHX2	*Bra039469*	GCTTTTGTGTTTCCTCTTTCCTTCT	TCGTCGTGCTTTGATGGGG
BrNHX3	*Bra002905*	ACCATCGTCGTTGTCCTCTTTAC	AGCGTCGCATCTTCCTTCG
BrNHX4	*Bra020755*	CACCGACATCATCTTTCCACG	ACTACCTCTGAAACGCCACGG
BrNHX6	*Bra035130*	TTTGCTCTTGCTCTACAATCTGTTC	CGGCTATCGCCTACAACCTCT
BrNHX7	*Bra017430*	GCTTAGGCAACAATCACTCGC	TATCTGAGGACGCAGGAGTTTGA
BrNHX8	*Bra026197*	GCTGTGTCCCTATCACTCGCTC	GTATTCTTTTCTTGGTGCCCGTT

2.6. Statistical analysis

All data were analyzed using the statistical program package SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance and the SSR (shortest significant ranges) tests were used to analyze the differences among the salt-treatments. Means were separated by different letters at $P < 0.05$ level.

3. Results and discussion

3.1. The growth of Chinese cabbage under salt stress

Although Chinese cabbage has long-term viability under 200 mM NaCl, it can not produce seeds (Qiu et al., 2015). Hence, Chinese cabbage cannot be regarded as a halophyte (Yuan et al., 2019). Salt treatments (100–400 mM NaCl) led to significant decreases in fresh weight of individual plant of these two Chinese cabbage cultivars. The fresh weight of QMY plants under 100, 200, 300, 400 mM NaCl was 56.3%, 39.4%, 21.7% and 16.9% of that of the control plants, respectively. The growth of KC-38 plants was inhibited more than that of QMY, which fresh weight was 49.6%, 37.0%, 17.9% and 13.6% of that of the control plants, respectively (Fig. 1a). In addition, the seedlings of KC-38 were yellowed and wilted under 400 mM NaCl (Fig. 1c). The common feature of these two cabbage cultivars was that the ratio of root/shoot was increased after salt treatment (Fig. 1b), indicating that the growth of the shoots of Chinese cabbage was inhibited more significantly by the salt stress compared with the roots. The higher root/shoot ratio in KC-38 suggested that the shoot of KC-38 was more sensitive to salt stress than that of QMY, which means QMY has better salt tolerance than KC-38.

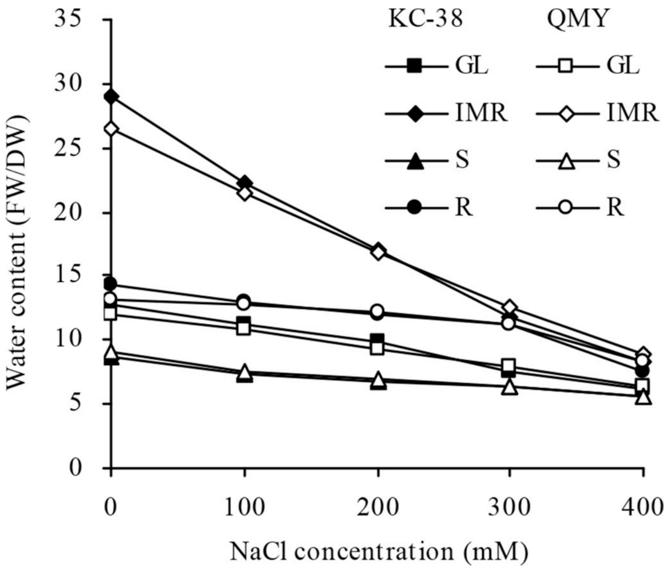

Fig. 2. The water content (FW/DW) of green leaves (GL), inflated midribs (IMR), stems (S) and roots (R) of Chinese cabbage seedlings exposed to 100–400 mM NaCl for 30 days. The data are means of ten replicates.

Chinese cabbage is a juicy vegetable, characterized by a large amount of water stored in its succulent midrib. Under the control condition, the water contents (FW/DW) in the midrib of KC-38 and QMY were 28.96 and 26.64, respectively. The absolute water contents are 96.5% and 96.2%, respectively (Fig. 2). With the increase of NaCl concentration, the water content of all organs of the two Chinese cabbage cultivars gradually decreased, and the water content of the leaves (including green leaves and inflated midribs) decreased more than that

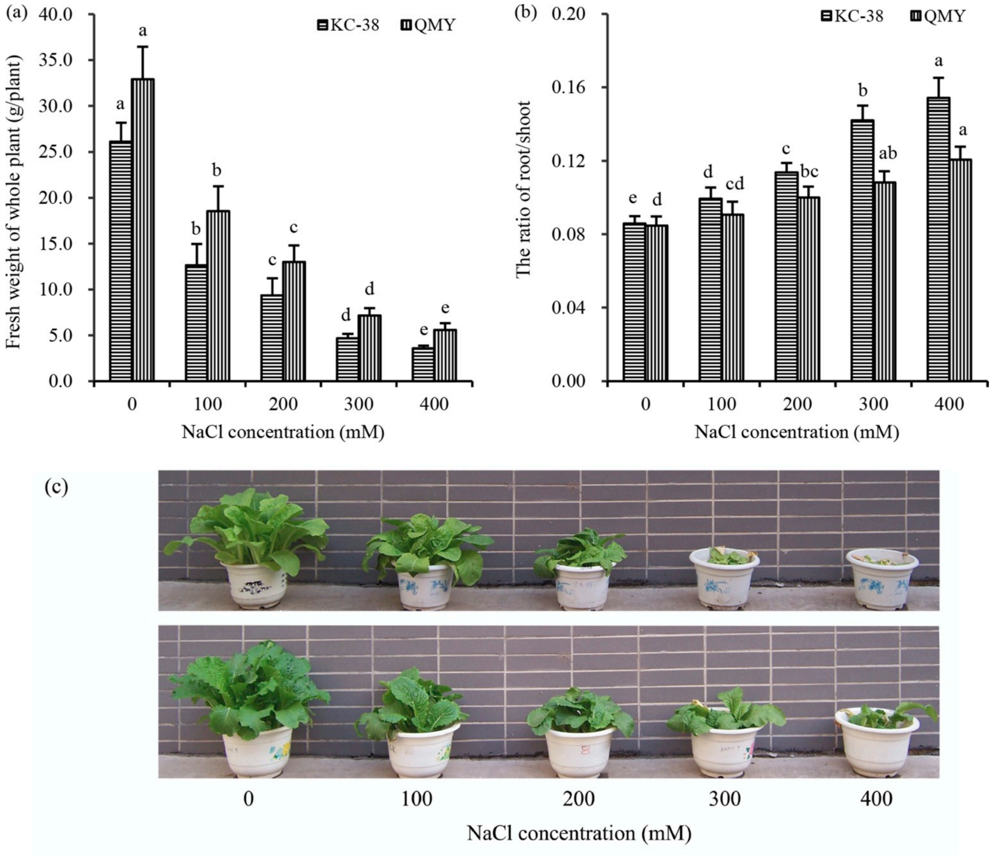

Fig. 1. The fresh weight (a), root/shoot ratio (b) and growth phenotypes (c) of Chinese cabbage seedlings exposed to 100–400 mM NaCl for 30 days. The data are means ± SD of ten replicates. Different letters show significant differences with $P < 0.05$.

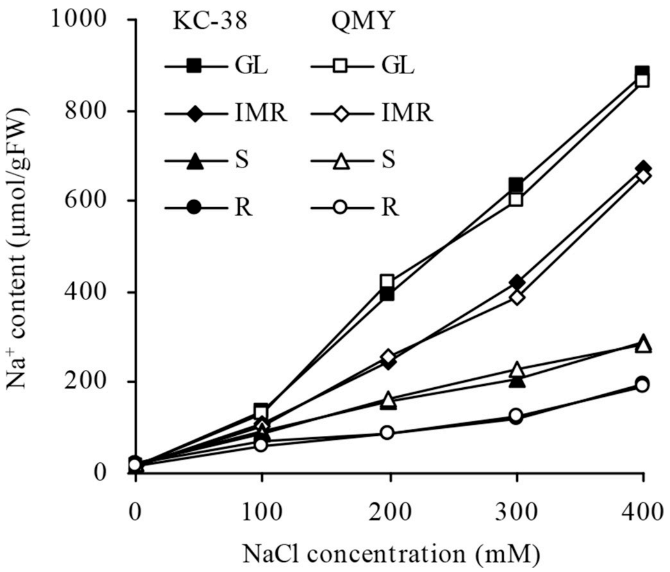

Fig. 3. The Na^+ content (μmol/gFW) of green leaves (GL), inflated midribs (IMR), stems (S) and roots (R) of Chinese cabbage exposed to 100–400 mM NaCl for 30 days. The data are means of five replicates.

of the roots. The water contents in the midribs of KC-38 and QMY, treated by 400 mM NaCl for 30 days, were only 28.5% and 33.6% of that of the control midribs, respectively. Therefore, the osmotic stress caused by salt on Chinese cabbage leaves was significantly greater than that on the stems and roots. The reduced water content in the salt-treated organs suggested that Chinese cabbage could not dilute salt by absorbing large amounts of water, unlike euhalophytes (Song and Wang, 2015). After salt treatment, the water contents of the crop plants all reduced. (Rus et al., 2005). Increasing the cell sap concentration by reducing the water content could help plants adapt to the osmotic stress caused by salinity (Hamouda et al., 2016).

3.2. Na^+ distribution in plants

The Na^+ contents (μmol/gFW) in green leaves, inflated midribs, stems and roots of KC-38 and QMY all increased markedly with the increase of NaCl-treatment concentration (Fig. 3). The Na^+ contents and their increasing trend in the organs of these two Chinese cabbage cultivars were similar after salt-treatment. The Na^+ content was in the following order, green leaves > inflated midribs > stems > roots, under the same NaCl concentration. The Na^+ content in the green leaves was about 2 times that of the roots under 100 mM NaCl condition. However, the Na^+ content in the green leaves was more than 4 times that of the roots under the condition of 200–400 mM NaCl. The Na^+ distribution trend was consistent with the flow direction of the transpiration stream. Na^+ eventually flowed to the green leaves and accumulated there. After exposure to 400 mM NaCl for 30 days, the Na^+ content in the green leaves of these two Chinese cabbage cultivars exceeded 850 μmol/gFW, which is equivalent to the leaf Na^+ level of *Suaeda salsa*, an euhalophyte cultivated under 400 mM NaCl for 30 days (Song and Wang, 2015). While the stems of seawater-treated cabbage (*Brassica oleracea* L.) showed the highest Na^+ content, following by the leaves and the roots (Gu et al., 2016). The mode of Na^+ distribution was even different in the same genus plants. Both Chinese cabbage and *B. oleracea* could not block salt in the roots. Some halophytes, such as reeds, have a significantly higher salt content in the roots than in the shoots of the plant, which accumulate salt in the roots to relieve the salt damage to the shoot (Fujimaki et al., 2015). Chinese cabbage preferentially accumulates salt into the leaves, reducing salt-damage to the roots. The Na^+-distribution strategy of cabbage is similar to wheat (Rahnama et al., 2011) and cotton (Peng et al., 2016).

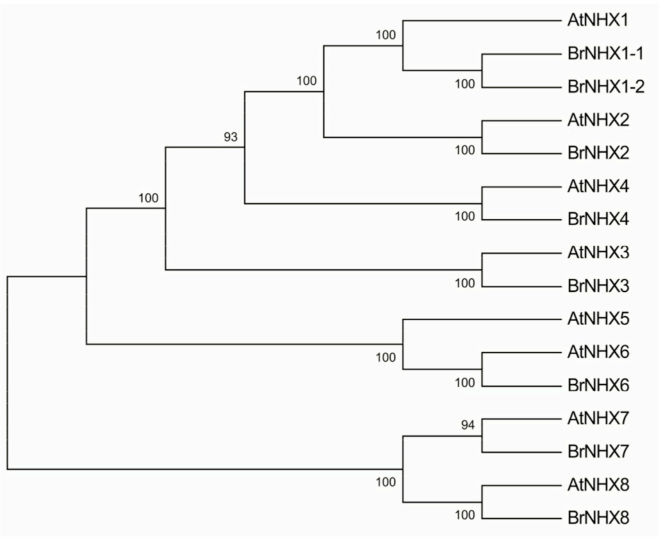

Fig. 5. Phylogenetic relationships analysis of NHXs between Chinese cabbage and *A. thaliana*.

Since the Na^+ concentrations in the organs of the salt-treated Chinese cabbage, especially the leaves, were very high (Fig. 3), Na^+ sequestration should be an important cellular strategy for Chinese cabbage plants. The data in Fig. 4 show that the distribution of Na^+ in apoplast and protoplast of the leaves of these two Chinese cabbage

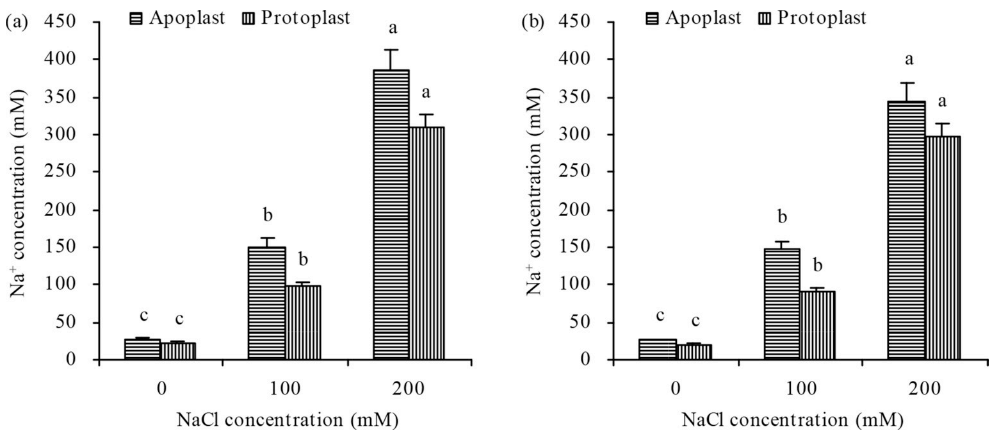

Fig. 4. The Na^+ concentration in the apoplast and protoplast of the leaves of KC-38 (a) and QMY (b) seedlings exposed to 100–400 mM NaCl for 30 days. The data are means of five replicates.

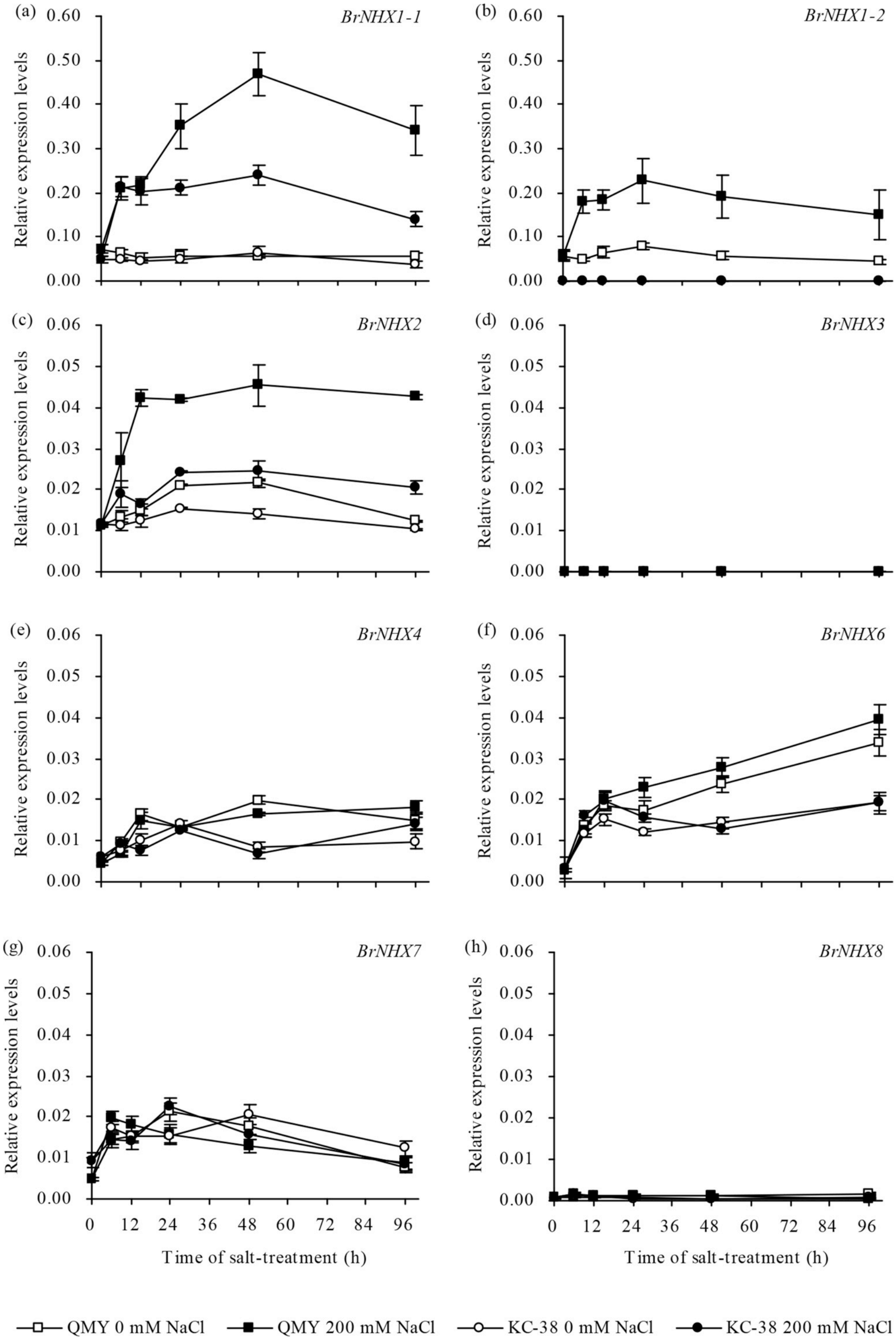

Fig. 6. Expression patterns of *BrNHX* genes involved in sodium segregation in leaves of Chinese cabbage seedlings exposed to 0 and 200 mM NaCl for 0–96 h. The gene expression levels were calculated by the comparative $2^{-\Delta\Delta C_T}$ method with *BrACTIN* as an internal control. Each sample was repeated three times.

cultivars also was similar. The Na^+ concentration in the protoplast was lower than that in the apoplast, suggesting that either Na^+ was passively absorbed by the leaf cell, or leaf cells excreted the excessive Na^+ out of the protoplast. In contrast to Chinese cabbage, the Na^+ concentration in the protoplast of leaves was higher than that of the apoplast in *Suaeda salsa* under saline condition (Qiu et al., 2007). This is because *S. salsa* plants are addicted to NaCl and use Na^+ as the main osmotic regulator. The Na^+ concentrations in both apoplast and protoplast of Chinese cabbage leaves treated with 200 mM NaCl were as high as 300–400 mM. High concentrations of Na^+ are toxic to the cytoplasm. Plants must either remove Na^+ out of the cells or sequester Na^+ into vacuoles by Na^+/H^+ antiporter (Flower and Colmer, 2015; Hasegawa, 2013).

3.3. Phylogenetic relationships of BrNHXs

A total of eight BrNHX family members were identified in the Chinese cabbage genome by a genome-wide analysis, according to the conserved amino acid sequence "FFIYLLPPI" of the NHX protein and the gene sequence of *AtNHXs* (An et al., 2007). The sequences of BrNHX members were downloaded from the *Brassica* database (http://brassicadb.org/brad/) (Wang et al., 2011). In order to understand the classification of the *BrNHX* genes in Chinese cabbage, a phylogenetic tree was constructed based on the full-length protein sequences of BrNHXs and AtNHXs by using the bootstrap–neighbor-joining method. The BrNHX members were named by the orthologs of *A. thaliana* based on the similarity of the protein sequences (Fig. 5). Because both *A. thaliana* and Chinese cabbage belong to *Brassicaceae*, their NHX members were highly homologous. Among the BrNHXs, both BrNHX1-1 and BrNHX1 - 2 were highly homologous to AtNHX1, and no member highly homologous to AtNHX5 was found. BrNHX2 - 8 were highly homologous to the respective AtNHX member. It was speculated that the BrNHX members and their homologous AtNHX members should have similar physiological function (Dong et al., 2018). There are 6 and 5 NHX members were identified in maize (ZmNHX1-6, Zörb et al., 2005) and rice (OsNHX1-5, Fukuda et al., 2011), respectively. However, A total of 35 NHX proteins were identified in wheat recently [TaNHX1-12(A, B, C, or D), Sharma et al., 2019]. These NHX proteins control pH and cation homeostasis and are localized within vacuole, plasma and organelle membranes (Chanroj et al., 2012).

3.4. *Expression patterns of BrNHX genes*

Na^+/H^+ antiporter is the key factors determining the capacity for Na^+ compartmentalization in leaves (Hasegawa, 2013; Peng et al., 2016). AtNHX1, AtNHX2 and AtNHX5, as the salt tolerance determinants in *A. thaliana*, have been proven to play the major role in Na^+ compartmentalization (Yokoi et al., 2002; Dong et al., 2018). In order to further elucidate the mechanism of Na^+ segregation in Chinese cabbage leaves, the expression patterns of the *BrNHX* genes involving in Na^+ segregation were analyzed with RT-qPCR (Fig. 6). Among the *BrNHX* genes, *BrNHX1-1* gene had the highest expression level (Fig. 6ab). However, *BrNHX1-2* was only expressed in QMY (Fig. 6b). The *BrNHX1-1* gene was constitutively expressed in Chinese cabbage leaves, and its expression was increased after salt treatment. After a 48-h exposure to 200 mM NaCl, the expression level of *BrNHX1-1* reached the maximum in QMY leaves, which was about 8 times that of the control. After a 24-h exposure to 200 mM NaCl, the expression level of *BrNHX1-2* reached the maximum in the QMY leaves, which was about 3 times that of the control. Because *BrNHX1-1* and *BrNHX1-2* encode tonoplast Na^+/H^+ antiporters, it is speculated that the leaves of Chinese cabbage should have a strong ability of sequestering Na^+ into vacuoles. The expression level of *BrNHX2* gene, encoding another tonoplast Na^+/H^+ antiporter, was also increased about 1 fold in both KC-38 and QMY leaves after salt treatments, while the expression level was only one-tenth of that of *BrNHX1-1* (Fig. 6c). The expression levels of other *BrNHX* genes, including *BrNHX7* (encoding a Na^+/H^+ antiporter in the plasma membrane), were relatively low, and not increased obviously after salt treatment (Fig. 6 d-h). Taken together, these results implicate that BrNHX1-1 and BrNHX1-2, as salt tolerance determinants, play a major role in vacuolar compartmentalization of Na^+ in Chinese cabbage leaves. BrNHX2 might play a less important role in salt-tolerance compared with BrNHX1-1 and BrNHX1-2.

It has been documented that constitutive expression of the *AtNHX3* gene can also increase the salt tolerance of sugar beet (Liu et al., 2008). So, *BrNHX3* and other *BrNHX* gene members may also play a role in other organs or other developmental stages of Chinese cabbage. In addition, NHXs ars not only the functional antiporter responsible for Na^+/H^+ exchange, but also for H^+-linked K^+ transport into vacuoles, and thereby enhance the salt tolerance of plants (Jiang et al., 2010; Fukuda et al., 2011; Zhang et al., 2015).

4. Conclusions

Our results showed that though Chinese cabbage plants could survive the 400 mM-NaCl stress for 30 days, the salt-treated plants had smaller biomass and lower succulent leaves compared with the plants under the normal conditions. The Na^+ content in the organs of Chinese cabbage plants tended to increase with the increasing NaCl-treatment concentration. The Na^+ content in the organs was in the following order: green leaves > inflated midribs > stems > roots, in the salt-treated plants. The Na^+ concentration in apoplast was higher than that in protoplast of the green leaves. These results indicated that Na^+ was passively transported into the leaves of Chinese cabbage along the transpiration stream. The capacity of Na^+ compartmentation into vacuole under salt stress was improved due to the elevated expression of *BrNHX1-1*, *BrNHX1-2* and *BrNHX2*. The salt tolerance of QMY was slightly better than KC-38, which may be related to the expression levels of these three *BrNHX* genes. This study on salt responses of Chinese cabbage cultivars, KC-38 and QMY, should contribute to a broad understanding of salt adaptation in vegetable plants.

Author contributions

M. Chen and B.S. Wang conceived and designed the research. J. Wang, P. Wang, W.R. Zhang and X.Y. Yang performed experiments. N.W. Qiu and J.K. Sun analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 41871089), the Open Research Fund Program of Shandong Provincial Key Laboratory of Eco-Environmental Science for Yellow River Delta (Projects No. 2018KFJJ01) and the Open Fund of Shandong Provincial Key Laboratory of Plant Stress (Projects No. KF201801).

References

An, R., Chen, Q.J., Chai, M.F., Lu, P.L., Su, Z., Qin, Z.X., Chen, J., Wang, X.C., 2007. *AtNHX8*, a member of the monovalent cation:proton antiporter-1 family in *Arabidopsis thaliana*, encodes a putative Li^+/H^+ antiporter. Plant J. 49, 718–728. https://doi.org/10.1111/j.1365-313X.2006.02990.x.

Chanroj, S., Wang, G., Venema, K., Zhang, M.W., Delwiche, C.F., Sze, H., 2012. Conserved and diversified gene families of monovalent cation/H^+ antiporters from algae to flowering plants. Front. Plant Sci. 3, 25. https://doi.org/10.3389/fpls.2012.00025.

Chen, X.G., Lu, X.K., Shu, N., Wang, D.L., Wang, S., Wang, J.J., Guo, L.X., Guo, X.N., Fan, W.L., Lin, Z.X., Ye, W.W., 2017. *GhSOS1*, a plasma membrane Na^+/H^+ antiporter gene from upland cotton, enhances salt tolerance in transgenic *Arabidopsis thaliana*. PLoS One 12, e0181450. https://doi.org/10.1371/journal.pone.0181450.

Deinlein, U., Stephan, A.B., Horie, T., Luo, W., Xu, G.H., Schroeder, J.I., 2014. Plant salt-tolerance mechanisms. Trends Plant Sci. 19, 371–379. https://doi.org/10.1016/j.tplants.2014.02.001.

Dong, W., Li, D.L., Qiu, N.W., Song, Y.G., 2018. The functions of plant cation/proton antiporters. Biol. Plant. 62, 421–427. https://doi.org/10.1007/s10535-018-0790-7.

Flower, T.J., Colmer, T.D., 2015. Plant salt tolerance: adaptations in halophytes. Ann. Bot-London 115, 327–331. https://doi.org/10.1093/aob/mcu267.

Flowers, T.J., Yeo, A.R., 1986. Ion relations of plants under drought and salinity. Funct. Plant Biol. 13, 75–91. https://doi.org/10.1071/pp9860075.

Food and Agriculture Organization of the United Nations, 2009. Increasing Crop Production Sustainably. The Perspective Biological Processes, " ed. Department of Economic and Social Development (Rome: FAO). http://www.fao.org/land-water/en/.

Fujimaki, S., Maruyama, T., Suzui, N., Kawachi, N., Miwa, E., Higuchi, K., 2015. Base to tip and long-distance transport of sodium in the root of common reed [*Phragmites australis* (Cav.) Trin. ex Steud.] at steady state under constant high-salt conditions. Plant Cell Physiol. 56, 943–950. https://doi.org/10.1093/pcp/pcv021.

Fukuda, A., Nakamura, A., Hara, N., Toki, S., Tanaka, Y., 2011. Molecular and functional analyses of rice NHX-type Na^+/H^+ antiporter genes. Planta 233, 175–188. https://doi.org/10.1007/s00425-010-1289-4.

Gu, M.F., Li, N., Shao, T.Y., Long, X.H., Brestic, M., Shao, H.B., Li, J.B., Mbarki, S., 2016. Accumulation capacity of ions in cabbage (*Brassica oleracea* L.) supplied with sea water. Plant Soil Environ. 62, 314–320. https://doi.org/10.17221/771/2015-PSE.

Hamouda, I., Badri, M., Mejri, M., Cruz, C., Siddique, K.H.M., Hessini, K., 2016. Salt tolerance of *Beta macrocarpa* is associated with efficient osmotic adjustment and increased apoplastic water content. Plant Biol. 18, 369–375. https://doi.org/10.1111/plb.12419.

Hasegawa, P.M., 2013. Sodium (Na^+) homeostasis and salt tolerance of plants. Environ. Exp. Bot. 92, 19–31. https://doi.org/10.1016/j.envexpbot.2013.03.001.

Jiang, X.Y., Leidi, E.O., Pardo, J.M., 2010. How do vacuolar NHX exchangers function in plant salt tolerance? Plant Signal. Behav. 5, 792–795. https://doi.org/10.4161/psb.5.7.11767.

Kumar, M., Choi, J.Y., Kumari, N., Pareek, A., Kim, S.R., 2015. Molecular breeding in *Brassica* for salt tolerance: importance of microsatellite (SSR) markers for molecular breeding in *Brassica*. Front. Plant Sci. 6, 688. https://doi.org/10.3389/fpls.2015.00688.

Liu, H., Wang, Q.Q., Yu, M.M., Zhang, Y.Y., Wu, Y.B., Zhang, H.X., 2008. Transgenic salt-tolerant sugar beet (Beta vulgaris L.) constitutively expressing an Arabidopsis thaliana vacuolar Na^+/H^+ antiporter gene, AtNHX3, accumulates more soluble sugar but less salt in storage roots. Plant Cell Environ 31, 1325–1334. https://doi.org/10.1111/j.1365-3040.2008.01838.x.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59, 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911.

Peng, Z., He, S.P., Sun, J.L., Pan, Z.E., Gong, W.F., Lu, Y.L., Du, X.M., 2016. Na^+ compartmentalization related to salinity stress tolerance in upland cotton (*Gossypium hirsutum*) seedlings. Sci. Rep. 6, 34548. https://doi.org/10.1038/srep34548.

Qiu, N.W., Chen, M., Guo, J.R., Bao, H.Y., Ma, X.L., Wang, B.S., 2007. Coordinate up-regulation of V-H^+-ATPase and vacuolar Na^+/H^+ antiporter as a response to NaCl treatment in a C_3 halophyte *Suaeda salsa*. Plant Sci. 172, 1218–1225. https://doi.org/10.1016/j.plantsci.2007.02.013.

Qiu, N.W., Liu, Q., Wang, F.D., Zhao, N., Sun, K.Y., Miao, X.M., Zhao, L.J., Li, L.L., Gao, J.W., 2015. Long-term observation on salt tolerance of Chinese cabbage at different development stages. Plant Physiol. J. 51, 1597–1603. (in Chinese with English Abstract). https://doi.org/10.13592/j.cnki.ppj.2015.1007.

Qiu, N.W., Liu, Q., Li, J.J., Zhang, Y.H., Wang, F.D., Gao, J.W., 2017. Physiological and transcriptomic responses of Chinese cabbage (*Brassica rapa* L. ssp. *Pekinensis*) to salt stress. Int. J. Mol. Sci. 18, 1953. https://doi.org/10.3390/ijms18091953.

Rahnama, A., Poustini, K., Tavakkol-Afshar, R., Ahmadi, A., Alizadeh, H., 2011. Growth properties and ion distribution in different tissues of bread wheat genotypes (*Triticum aestivum* L.) differing in salt tolerance. J. Agron. Crop Sci. 197, 21–30. https://doi.org/10.1111/j.1439-037X.2010.00437.x.

Rus, A.M., Bressan, R.A., Hasegawa, P.M., 2005. Unraveling salt tolerance in crops. Nat. Genet. 37, 1029–1030. https://doi.org/10.1038/ng1005-1029.

Saha, G., Park, J., Jung, H., Ahmed, N.U., Kayum, M.A., Chung, M., Hur, Y., Cho, Y., Watanabe, M., Nou, I., 2015. Genome-wide identification and characterization of MADS-box family genes related to organ development and stress resistance in Brassica rapa. BMC Genomics 16, 178–198. https://doi.org/10.1186/s12864-015-1349-z.

Shah, N., Anwar, S., Xu, J.J., Hou, Z.K., Salah, A., Khan, S., Gong, J.F., Shang, Z.W., Qian, L., Zhang, C.Y., 2018. The response of transgenic *Brassica* species to salt stress: a review. Biotechnol. Lett. 40, 1159–1165. https://doi.org/10.1007/s10529-018-2570-z.

Sharma, H., Taneja, M., Upadhyay, S.K., 2019. Identification, characterization and expression profiling of cation-proton antiporter superfamily in *Triticum aestivum* L. and functional analysis of *TaNHX4-B*. Genomics. online. https://doi.org/10.1016/j.ygeno.2019.02.015 111.

Song, J., Wang, B.S., 2015. Using euhalophytes to understand salt tolerance and to develop saline agriculture: *Suaeda salsa* as a promising model. Ann. Bot-London. 115, 541–553. https://doi.org/10.1093/aob/mcu194.

Tang, X.L., Mu, X.M., Shao, H.B., Wang, H.Y., Brestic, M., 2015. Global plant-responding mechanisms to salt stress: physiological and molecular levels and implications in biotechnology. Crit. Rev. Biotechnol. 35, 425–437. https://doi.org/10.3109/07388551.2014.889080.

Tetlow, I.J., Farrar, J.F., 1993. Apoplastic sugar concentration and pH in barley leaves infected with brown rust. J. Exp. Bot. 44, 929–936. https://doi.org/10.1093/jxb/44.5.929.

Wang, X.W., Wang, H.Z., Wang, J., Sun, R.F., Wu, J., Liu, S.Y., Bai, Y.Q., Mun, J.H., Bancroft, I., Cheng, F., et al., 2011. The *Brassica rapa* Genome Sequencing Project Consortium: the genome of the mesopolyploid crop species *Brassica rapa*. Nat. Genet. 43, 1035–1039. https://doi.org/10.1038/ng.919.

Xu, Y.Y., Zhou, Y., Hong, S., Xia, Z.H., Cui, D.Q., Guo, J.C., Xu, H.X., Jiang, X.Y., 2013. Functional characterization of a wheat NHX antiporter gene *TaNHX2* that encodes a K^+/H^+ exchanger. PLoS One 8, e78098. https://doi.org/10.1371/journal.pone.0078098.

Yang, F.B., Yang, X.G., Xue, M.D., Huang, X.C., Li, B., Cui, X.X., Li, S., Qiu, N.W., Gao, J.W., 2017. Screening of salt tolerant varieties from eighteen Chinese cabbages. Shandong Agr. Sci. 49, 31–36 (in Chinese with English Abstract).

Yokoi, S., Quintero, F.J., Cubero, B., Ruiz, M.T., Bressan, R.A., Hasegawa, P.M., Pardo, J.M., 2002. Differential expression and function of *Arabidopsis thaliana* NHX Na^+/H^+ antiporters in the salt stress response. Plant J. 30, 529–539. https://doi.org/10.1046/j.1365-313X.2002.01309.x.

Yuan, F., Leng, B.Y., Wang, B.S., 2016. Progress in studying salt secretion from the salt glands in recretohalophytes: how Do Plants Secrete Salt? Front. Plant Sci. 7, 977. https://doi.org/10.3389/fpls.2016.00977.

Yuan, F., Leng, B.Y., Wang, B.S., 2019. Reproductive physiology of halophytes: current standing. Front. Plant Sci. 9, 1954. https://doi.org/10.3389/fpls.2018.01954.

Zhang, X.K., Lu, G.Y., Long, W.X., Zou, X.L., Li, F., Nishio, T., 2014. Recent progress in drought and salt tolerance studies in Brassica crops. Breed Sci. 64, 60–73. https://doi.org/10.1270/jsbbs.64.60.

Zhang, Y.M., Zhang, H.M., Liu, Z.H., Li, H.C., Guo, X.L., Li, G.L., 2015. The wheat NHX antiporter gene *TaNHX2* confers salt tolerance in transgenic alfalfa by increasing the retention capacity of intracellular potassium. Plant Mol. Biol. 87, 317–327. https://doi.org10.1007/s11103-014-0278-6.

Zhang, Y., Xu, A.X., Lang, L.N., Wang, Y., Liu, X., Liang, F.H., Zhang, B.B., Qin, M.F., Dalelhan, J., Huang, Z., 2018. Genetic mapping of a lobed-leaf gene associated with salt tolerance in *Brassica napus* L. Plant Sci. 269, 75–84. https://doi.org/10.1016/j.plantsci.2018.01.005.

Zörb, C., Noll, A., Karl, S., Leib, K., Yan, F., Schubert, S., 2005. Molecular characterization of Na^+/H^+ antiporters (*ZmNHX*) of maize (*Zea mays* L.) and their expression under salt stress. J. Plant Physiol. 162, 55–66. https://doi.org/10.1016/j.jplph.2004.03.010.